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### Citation for published version:

Garrido, D, Alber, A, Kut, E, Chanteloup, NK, Lion, A, Trotereau, A, Dupont, J, Tedin, K, Kaspers, B, Vervelde, L, Trapp, S, Schouler, C & Guabiraba, R 2018, 'The role of type I interferons (IFNs) in the regulation of chicken macrophage inflammatory response to bacterial challenge', *Developmental and Comparative Immunology*, vol. 86, pp. 156-170. <https://doi.org/10.1016/j.dci.2018.04.025>

### Digital Object Identifier (DOI):

[10.1016/j.dci.2018.04.025](https://doi.org/10.1016/j.dci.2018.04.025)

### Link:

[Link to publication record in Edinburgh Research Explorer](#)

### Document Version:

Peer reviewed version

### Published In:

Developmental and Comparative Immunology

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# Accepted Manuscript

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PII: S0145-305X(18)30101-0

DOI: [10.1016/j.dci.2018.04.025](https://doi.org/10.1016/j.dci.2018.04.025)

Reference: DCI 3161

To appear in: *Developmental and Comparative Immunology*

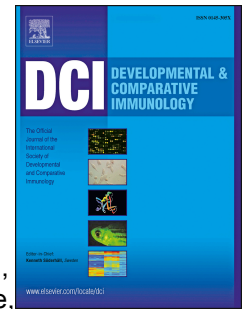
Received Date: 6 March 2018

Revised Date: 29 April 2018

Accepted Date: 29 April 2018

Please cite this article as: Garrido, D., Alber, A., Kut, E., Chanteloup, N.K., Lion, A., Trotereau, Angé., Dupont, Joë., Tedin, K., Kaspers, B., Vervelde, L., Trapp, S., Schouler, C., Guabiraba, R., The role of type I interferons (IFNs) in the regulation of chicken macrophage inflammatory response to bacterial challenge, *Developmental and Comparative Immunology* (2018), doi: 10.1016/j.dci.2018.04.025.

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**The role of type I interferons (IFNs) in the regulation of chicken  
macrophage inflammatory response to bacterial challenge**

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**Abstract**

Mammalian type I interferons (IFN $\alpha/\beta$ ) are known to modulate inflammatory processes in addition to their antiviral properties. Indeed, virus-induced type I interferons regulate the mammalian phagocyte immune response to bacteria during superinfections. However, it remains unresolved whether type I IFNs similarly impact the chicken macrophage immune response. We first evidenced that IFN $\alpha$  and IFN $\beta$  act differently in terms of gene expression stimulation and activation of intracellular signaling pathways in chicken macrophages. Next, we showed that priming of chicken macrophages with IFN $\alpha$  increased bacteria uptake, boosted bacterial-induced ROS/NO production and led to an increased transcriptional expression or production of *NOS2*/NO, *IL1B*/IL-1 $\beta$ , and notably *IFNB*/IFN $\beta$ . Neutralization of IFN $\beta$  during bacterial challenge limited IFN $\alpha$ -induced augmentation of the pro-inflammatory response. In conclusion, we demonstrated that type I IFNs differently regulate chicken macrophage functions and drive a pro-inflammatory response to bacterial challenge. These findings shed light on the diverse functions of type I IFNs in chicken macrophages.

**Keywords:** Avian pathogenic *E. coli*; chicken; inflammation; interferon stimulated genes; macrophages; type I interferons

## 1. Introduction

Interferons (IFNs) are key cytokines within the innate immune response. They were first discovered in 1957 due to their capacity to inhibit influenza virus replication in embryonated chicken eggs (Isaacs and Lindenmann, 1957). IFNs are divided into three subgroups: type I, II, and III IFNs. Type I IFNs, including various subtypes of IFN $\alpha$ , IFN $\beta$ , and some “minor” IFNs (i.e. IFN $\delta$ , IFN $\epsilon$ , IFN $\kappa$ , IFN $\tau$ , and IFN $\omega$ ), are produced during viral and bacterial infections (Bogdan et al., 2004; de Weerd and Nguyen, 2012; Ivashkiv and Donlin, 2014). In mammals, most cell types are able to produce IFN $\beta$ , including non-immune cells, while IFN $\alpha$  is mainly produced by hematopoietic cells, especially plasmacytoid dendritic cells (Ivashkiv and Donlin, 2014). IFN $\alpha$  and IFN $\beta$  bind to the same receptor, Interferon-alpha Receptor (IFNAR), which is composed of two subunits (IFNAR1 and IFNAR2) and expressed in the majority of tissues (de Weerd and Nguyen, 2012).

Binding of type I IFNs to IFNAR entails the rapid activation of different signalling pathways for the regulation of Interferon-Stimulated Genes (ISGs) (Hervas-Stubbs et al., 2011), many of which play a critical role in the limitation of viral replication (Schneider et al., 2014). In addition, type I IFNs have been shown to enhance antigen-presentation, regulate inflammasome activation and upregulate pro-inflammatory cytokines production in mammalian species (Hervas-Stubbs et al., 2011; Malireddi and Kanneganti, 2013; Simmons et al., 2012). In humans, dysregulated type I IFNs responses were shown to be associated to immune disorders such as chronic infection, autoimmune and inflammatory diseases (Ivashkiv and Donlin, 2014; Trinchieri, 2010). Therefore, a tight regulation is required to shape the outcome of type I IFN responses in order to achieve the balance between IFN-mediated protective immunity and exacerbated IFN signalling (Trinchieri, 2010).

Virus-induced type I IFNs have been associated to impaired host immune responses such as decreased bactericidal functions of phagocytic cells (Shepardson et al., 2016), granulocyte apoptosis (Merches et al., 2015; Navarini et al., 2006), over-activation of the Nod1/Nod2 pathway (Kim et al., 2011), decreased chemokine secretion (Nakamura et al., 2011; Shahangian et al., 2009), and attenuation of antimicrobial peptides expression (Lee et al., 2015). Consequently, certain cell populations such as macrophages may become affected by an enriched type I IFNs environment that is typical for viral infections (Shepardson et al., 2016). These cellular and molecular events are at the origin of the well-established principle that primary viral infections may predispose the host to bacterial superinfections (McCullers, 2014; Metzger and Sun, 2013). This observation is not restricted to humans and experimental mammalian models, since poultry species, including galliform birds, are often impacted by viral/bacterial co-infections (Ariaans et al., 2008; Gross, 1990; Kodihalli et al., 1994; Matthijs et al., 2009; Nakamura et al., 1994). However, cell populations and cytokines involved in the pathogenesis of co-infections affecting poultry are still poorly characterized.

In galliform birds, viral infections such as those caused by low pathogenic avian influenza virus (LPAIV) may lead to a type I IFN response (Adams et al., 2009; Cornelissen et al., 2012). However, it remains unclear whether this response contributes to predisposing animals to bacterial superinfection through a dysregulated macrophage function. Nevertheless, it is well established that macrophages play a key role during LPAIV infection and that they are efficiently responding to type I IFNs (Kodihalli et al., 1994; Qu et al., 2013). In turkeys, it has been demonstrated that LPAIV infection compromises pulmonary macrophages function, which would likely predispose birds to secondary bacterial infections (Kodihalli et al., 1994). These studies underscore the relevance of macrophages and their crucial role in the early phases of infection for the priming of an efficient antiviral host response to limit viral

dissemination (Abdul-Cader et al., 2017; Duan et al., 2017; Fujisawa et al., 1987). In addition, macrophages appear to have an important role in the control of avian colibacillosis, which is caused by avian pathogenic *E. coli* (APEC) strains (Guabiraba and Schouler, 2015; Mellata et al., 2003). Colibacillosis is the most relevant opportunistic bacterial infection of poultry. Its severity is frequently correlated to a primary viral infection, notably in regard to its pulmonary manifestation (Ariaans et al., 2008; Matthijs et al., 2009; Mosleh et al., 2017; Nakamura et al., 1994; Nolan et al., 2008; Umar et al., 2018; Umar et al., 2017).

In the present study, we provided first insights on how chicken macrophages respond to type I IFNs. In addition, we have set up an *in vitro* model based on IFN $\alpha$  priming of chicken macrophages followed by stimulation with *E. coli* lipopolysaccharide (LPS) or infection with avian pathogenic *E. coli* (APEC) to assess the impact of type I IFNs on the macrophage response to bacterial challenge. Our data provide new information on the cellular and molecular determinants of chicken macrophage functions in an inflammatory milieu likely to be encountered during viral/bacterial co-infection in poultry.

## 2. Materials and methods

### 2.1. Reagents

Lipopolysaccharide (LPS, from *E. coli* O55:B5) was purchased from Sigma-Aldrich, UK. Chicken IFN $\alpha$  and IFN $\beta$  were produced in *E. coli* and purified as previously described (Schultz et al., 1995a; Schultz et al., 1995b). Purified rabbit anti-chicken IFN $\beta$  antiserum was obtained as previously described (Schwarz et al., 2004). Recombinant chicken type I IFNs and IFN $\beta$  antiserum were tested negative for endotoxin contamination using HEK-Blue™ TLR4 cells designed for studying the stimulation of TLR4 by monitoring the activation of

NF- $\kappa$ B and AP-1 (InvivoGen, USA). SB-203580 (p38 MAP Kinase inhibitor) and Wortmannin (PI3-kinase/Akt inhibitor) were purchased from Tocris Bioscience, UK. BAY11-7082 (I $\kappa$ B- $\alpha$  inhibitor) and BX795 (TBK1/IKK $\epsilon$  inhibitor) were purchased from InvivoGen, USA. During the experiments, LPS, IFN $\alpha$ , IFN $\beta$  and IFN $\beta$ -antiserum were diluted in RPMI 1640 medium (Gibco, UK). Inhibitors were diluted in dimethyl sulfoxide (DMSO, Sigma-Aldrich, UK) and RPMI 1640 medium. Final concentration of DMSO in cell culture wells never exceeded 0.1%.

## 2.2. Cell culture

HD11, an avian myelocytomatosis virus (MC29)-transformed chicken macrophage-like cell line (Beug et al., 1979), was cultured in RPMI 1640 medium (Gibco, UK), supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco, UK), 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (GE Healthcare, USA). HD11 cells were routinely grown in 75-cm<sup>2</sup> flasks (Corning, USA) at 41°C and 5% CO<sub>2</sub>.

An HD11-NF $\kappa$ B luciferase reporter cell line was constructed by infection of cells with replication-incompetent, lentivirus-based pseudoviral particles harboring a vector containing a basal promoter element (TATA box) and tandem repeats of an NF $\kappa$ B consensus sequence fused to a luciferase reporter gene (Cignal Lenti Reporters, SABiosciences, Frederick, Maryland, USA). Cell lines expressing the reporter fusion were selected under puromycin selection according to the manufacturer's instructions, and individual clones purified by limited dilution. Clones were subsequently screened for NF $\kappa$ B activation in response to LPS and those showing high induction ratios with a low signal/noise ratio were retained. Cells were routinely cultured in DMEM F-12 (1:1) medium (Gibco, UK), supplemented with 10% heat-inactivated FCS, 15 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml



streptomycin and 5 µg/ml puromycin (Sigma-Aldrich, UK), and incubated as described above.

The CEC32-Mx-Luc and the CEC32-NFκB-Luc reporter cell lines are quail fibroblast cell lines carrying the luciferase gene under the control of chicken Mx promoter (Schwarz et al., 2004) or carrying an NFκB-regulated luciferase reporter gene (Gyorfy et al., 2003), respectively. CEC32-Mx-Luc and the CEC32-NFκB-Luc were kindly provided by Prof. Peter Stäheli (University of Freiburg, Germany). CEC32 luciferase reporter cells were cultivated in DMEM GlutaMAX™-I supplemented with 8% heat-inactivated FCS, 2% heat-inactivated chicken serum (Gibco, UK), 4.5 g/l D-glucose, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml geneticin (G418) (Gibco, UK) and grown in 25-cm<sup>2</sup> flasks (Corning, USA) at 41°C and 5% CO<sub>2</sub>.

Chicken bone marrow derived macrophages (chBMDM) were generated from bone marrow cells using recombinant chicken colony-stimulating factor 1 (CSF-1) (Garceau et al., 2010) produced in COS-7 cells (fibroblast-like cell line derived from monkey kidney tissue, ATCC, USA) transfected with a pTarget vector (Promega, UK) expressing chicken CSF-1 (kindly provided by Prof. Pete Kaiser, The Roslin Institute, UK). Briefly, femurs and tibias of 4 week-old White Leghorn B13/B13 histocompatible chickens were removed, both ends of the bones were cut and the bone marrow was flushed with RPMI 1640 supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were washed and re-suspended in RPMI 1640 medium then loaded onto an equal volume of Histopaque-1077 (Sigma-Aldrich, UK) and centrifuged at 400 g for 20 min. Cells at the interface were collected and washed twice in RPMI 1640 medium. Purified cells were seeded at 1x10<sup>6</sup> cells/ml in sterile 60 mm bacteriological petri dishes in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and COS-7 supernatant containing chicken CSF-1 at 41°C and 5% CO<sub>2</sub>. Half of the medium was

replaced with fresh medium containing chicken CSF-1 at day 3. At day 6 adherent cells were harvested and washed in phosphate buffered saline (PBS, Gibco, UK) supplemented with 2 mM Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, UK) and re-suspended in RPMI 1640 supplemented with 10% heat-inactivated FCS, 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Chicken lung macrophages were obtained from transgenic birds expressing the fluorescent mApple reporter under control of promoter and enhancer elements of the chicken CSF1R locus (Balic et al., 2014) as described previously (Jansen et al. 2010). Briefly, lungs from 4 week-old MacRed chickens were removed, cut into pieces, and incubated with a DNase I/collagenase A mix (1 mg/ml and 3 mg/ml, respectively; Sigma-Aldrich, UK) diluted in supplement free RPMI 1640 medium for 30 min at 41°C and 5% CO<sub>2</sub>. The digested tissue suspension was filtered through a 70 µm strainer, washed with PBS, and leukocytes were purified using a density gradient as described above. Cells at the interface were collected and washed twice in PBS. Cells were cultured at 1.5x10<sup>6</sup> cells/ml in 6-well plates in a final volume of 5 ml with RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 200 ng/ml of CSF-1 at 41°C and 5% CO<sub>2</sub> for 24h. The next day, culture medium containing non-adhered cells was removed and replaced with fresh complete RPMI 1640 medium without CSF-1 for the experimental treatment. The number of adherent macrophages was evaluated in parallel by flow cytometry and was approximately 5x10<sup>5</sup> cells per well.

### **2.3. *In vitro* stimulation protocol**

HD11 and chBMDM cells were seeded in 12-well plates at 5x10<sup>5</sup> cells/well and 7.5x10<sup>5</sup> cells/well, respectively, and incubated at 41°C and 5% CO<sub>2</sub> overnight prior to stimulation. Chicken lung macrophages at 5x10<sup>5</sup> cells/well were obtained as described above and not re-

seeded. Next, the cells were pretreated for 16h with chicken recombinant IFN $\alpha$  (50 ng/ml) unless otherwise indicated. Cells were gently washed with PBS prior to stimulation with IFN $\alpha$  (50 ng/ml), LPS (10 ng/ml), purified IFN $\beta$ -antiserum (17.5  $\mu$ g/ml) or IFN $\beta$  (50 ng/ml) at different treatment combinations and at different time-points. In all experiments, mock controls were treated with medium or medium with 0.1% DMSO (when pharmacological inhibitors were used). Supernatants were harvested and stored at -20°C after stimulation for further analysis. HD11 cells and chBMDM were washed in PBS and lysed with RNA lysis buffer (Macherey-Nagel, Germany) containing 2-mercaptoethanol (Merck Millipore, Germany), snap frozen in liquid nitrogen and stocked at -80°C until RNA extraction. Chicken lung macrophages were washed in PBS and lysed with RNA lysis buffer (Qiagen, Germany) containing 2-mercaptoethanol. For protein dosage and western blot analysis, cells were washed in PBS followed by cell lysis using Laemmli Sample Buffer (BioRad, USA) containing a proteases inhibitors cocktail (Santa Cruz Biotechnology, USA) and 2.5% 2-mercaptoethanol, and stocked at -20°C.

#### 2.4. Experimental design for *in vitro* infection

HD11 were seeded in 12-well plates at  $5 \times 10^5$  cells/well and chicken lung macrophages were used at a final number of  $5 \times 10^5$  cells/well in 6-well plates (in which they were obtained) in complete RPMI 1640 medium, and incubated at 41°C under 5% CO $_2$  overnight. The APEC strains used for infections were BEN2908 (O2:K1:H5), a nalidixic acid-resistant derivative of strain MT78 which was isolated from the trachea of a chicken with respiratory infection (Dho and Lafont, 1982) or BEN2908 harboring pFPV25.1 (a plasmid expressing GFP) (Valdivia and Falkow, 1996). Bacteria were diluted at the appropriate concentration in supplement-free RPMI 1640 medium, and cells were infected at a multiplicity of infection (MOI) of 10 followed by incubation at 41°C under 5% CO $_2$ . The mock control group received supplement-free RPMI 1640 medium without bacteria. After 1h (adhesion period), one group of APEC-

infected HD11 cells were washed with PBS then lysed with PBS containing 0.1% Triton X-100 (Sigma-Aldrich, UK). Bacteria in the cell lysates were plated onto LB agar plates to evaluate the number of adherent bacteria (colony-forming units). For the other groups, HD11 or chicken lung macrophages were gently washed with PBS and remaining extracellular bacteria were killed by incubating cells with FCS-free medium containing gentamicin (100 µg/ml) for 1h 30 min. Cells were then lysed with 1X PBS containing 0.1% Triton X-100 or harvested in FACS buffer (PBS supplemented with 2% heat-inactivated FCS and 2 mM EDTA) for further flow cytometry analysis. Remaining intracellular viable bacteria were plated onto LB agar to determine the number of intracellular bacteria. For gene expression analysis, an additional group of HD11 cells were infected as described above, and incubated with medium containing gentamicin (10 µg/ml) for 6h. Cells were washed with PBS then lysed with RNA lysis buffer (Macherey-Nagel, Germany).

## 2.5. Flow cytometry analysis

Cell viability following different stimuli was assessed using the chicken Annexin V Fluorescein kit (Kingfisher Biotech, USA) and the fluorescent DNA intercalator 7-aminoactinomycin D (7-AAD, BD Biosciences, USA) as markers of apoptosis and necrosis, respectively. HD11 cells were seeded at  $2 \times 10^5$  cells/well in 24-well plates and pretreated with IFN $\alpha$  (50 ng/ml) (unless otherwise indicated). Following stimulation with IFN $\alpha$  (50 ng/ml) or LPS (10 ng/ml) for 6h, supernatants were discarded and the cells were harvested and washed in PBS. Cells were stained according to the manufacturer's protocol and the viability was analyzed by flow cytometry (BD FACS Calibur). Data were expressed as the percentage of cells undergoing late apoptosis/necrosis (Annexin V<sup>+</sup> 7-AAD<sup>+</sup>) over total acquired events (50,000 cells).

Expression of co-stimulation markers on the chicken macrophage HD11 cell line was also assessed. HD11 cells were treated as described above and harvested in FACS buffer (PBS supplemented with 2% heat-inactivated FCS and 2 mM EDTA). Cells were washed and incubated for 45 min at 4°C with a mouse anti-chicken CD40 antibody (BioRad, USA) at a 1:200 dilution or with the respective isotype control (BioRad) at a 1:200 dilution. Cells were again washed and incubated for 45 min with a rat anti-mouse IgG-specific secondary antibody coupled to fluorescein isothiocyanate (FITC, Thermo Fisher Scientific, USA). In a separate staining protocol, cells were also stained with a mouse anti-chicken MHC class II FITC (BioRad) or with the respective isotype control (BioRad) at a 1:200 dilution for 45 min. HD11 cells were washed and re-suspended in FACS buffer prior to analysis. Data were expressed as Mean Fluorescence Intensity (MFI) from FITC<sup>+</sup> cell populations over total acquired events (50,000 cells).

Bacterial fluorescence was assessed by flow cytometry. Briefly, chicken lung macrophages (5x10<sup>5</sup> cells/well in 6-well plates) were pretreated with the different stimuli and infected as described above. Lung macrophages were detached from the plate using TrypLE Express (Invitrogen, USA) for 15 min, washed, and harvested in FACS buffer prior to analysis (BD LSRFortessa™). SYTOX Blue Dead Cell Stain (Invitrogen, USA) was added to discriminate live and dead cells. Data were expressed as Mean Fluorescence Intensity (MFI) from GFP<sup>+</sup> cell populations over total acquired events (50,000 live cells).

## **2.6. Gene expression analysis**

Total RNA from HD11 cells and chBMDM was extracted from frozen cell lysates using the NucleoSpin<sup>®</sup> RNA kit (Macherey-Nagel, Germany) according to the manufacturer's instructions, while total RNA from lung macrophages was extracted using the RNeasy<sup>®</sup> Mini-Kit (Qiagen, Germany). Both protocols contained a DNase treatment step. RNA quality and

concentration were determined by NanoDrop spectrophotometric measurement (Thermo Scientific, USA). Total RNA (up to 1 µg per reaction) was reverse transcribed using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, USA). Quantitative Real-time PCR (qRT-PCR) was performed on a CFX96 machine (Bio-Rad, USA). The reaction mixture was composed of cDNA, iQ SYBR Green Supermix (Bio-Rad, USA), primer pairs (Eurogentec, Belgium) and nuclease-free water (Sigma-Aldrich, UK) in a total volume of 10 µl. qRT-PCR data were analyzed using the CFX Manager software 3.1 (Bio-Rad, USA). Gene expression for each target gene was normalized to gene expression levels of chicken hypoxanthine-guanine phosphoribosyltransferase (HPRT),  $\beta$ -2-microglobulin ( $\beta$ 2M) and/or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A list of primer pairs utilized in the present study is given in **Table 1**. Relative normalized expression was calculated using the  $2^{-\Delta\Delta C_t}$  method and data are represented as fold increase as compared to control (or mock) groups. Baseline cycle threshold ( $C_t$ ) values for the target genes in HD11 cells, chBMDM and lung macrophages are shown in **Supplementary Table 1**.

## **2.7. NO and ROS production**

Nitrite ( $\text{NaNO}_2$ ) concentration, as an index of nitric oxide (NO) production, was determined by spectrophotometry in cell culture supernatants using a standard Griess assay according to the manufacturer's instructions (Promega, UK). The absorbance was read at 550 nm in a Multiskan Ascent plate reader (Thermo Fisher Scientific, USA). The nitrite concentration was calculated using a sodium nitrite standard curve.

ROS (Reactive Oxygen Species) production was evaluated by flow cytometry using the CellROX<sup>®</sup> Green Reagent kit (Invitrogen, USA) according to the manufacturer's instructions. Briefly, HD11 cells were seeded at  $2 \times 10^5$  cells/well in 24-well plates and pretreated with IFN $\alpha$  (50 ng/ml) for 16h (unless otherwise indicated). Next, cells were gently washed with

PBS then incubated with fresh medium containing LPS (10 ng/ml) or IFN $\alpha$  (50 ng/ml) for 6h before the addition of CellROX<sup>®</sup> Green Reagent for 30 min. Cells were washed, harvested in FACS buffer and ROS production was determined by flow cytometry analysis (BD FACS Calibur). Data were expressed as Mean Fluorescence Intensity (MFI) from green fluorescent<sup>+</sup> cell population over total acquired events (50,000 cells).

## 2.8. Western blot

Total protein was quantified using a Quick Start<sup>™</sup> Bradford Protein Assay (Bio-Rad, USA). 15  $\mu$ L of protein-containing lysates were separated on a 12% polyacrylamide gel in Tris-Glycin-SDS buffer (25 mM, 200 mM and 0.5% respectively) and transferred to Porablot<sup>®</sup> nitrocellulose membranes (0.45  $\mu$ m) (Macherey-Nagel, Germany) using a Mini Trans-Blot<sup>®</sup> cell (Bio-Rad) in 1X CAPS (3-(cyclohexylamino)-1-propanesulfonic acid) buffer. Following overnight immersion at 4°C in a blocking solution (3% non-fat milk powder in buffer containing 10 mM Tris, 150 mM NaCl and 0.1% Tween 20), the membranes were washed and incubated for 1h at RT with a mouse anti-GAPDH antibody (MAB374, Millipore, USA) at a 1:500 dilution (3% milk powder in Tris-NaCl-Tween buffer), a rabbit anti-p38 antibody (#9212, Cell Signaling, USA) at a 1:1000 dilution, a rabbit anti-phospho-p38 antibody (#9211, Cell Signaling) at a 1:1000 dilution, a mouse anti-STAT1 antibody (1/Stat1, BD Bioscience, USA) at a 1:1000 dilution, a rabbit anti-phospho-STAT1 antibody (15H13L67, Life Technologies, USA) at a 1:1000 dilution, a rabbit anti-Akt (pan) antibody (#4691, Cell Signaling) at a 1:1000 dilution, a rabbit anti-phospho-Akt1/2/3 antibody (sc-7985-R, Santa Cruz Biotechnology) at a 1:1000 dilution, a mouse anti-phospho-I $\kappa$ B $\alpha$  (Ser32/36) antibody (#9246, Cell Signaling) at a 1:1000 dilution, a rabbit anti-p44/42 MAPK (Erk1/2) antibody (#4695, Cell Signaling) at a 1:1000 dilution or a rabbit anti-phospho-p44/42 MAPK (Erk1/2) antibody (#4377, Cell Signaling) at a 1:1000 dilution. Membranes were washed three times and incubated for 1h at RT with a mouse or rabbit IgG-specific secondary antibody coupled to



horseradish peroxidase (HRP, Sigma) at a dilution of 1:10000 (3% milk powder in Tris-NaCl-Tween buffer). After washing, the membranes were overlaid with the WesternBright® ECL peroxidase substrate (Advansta) and chemiluminescence was visualized using a Fusion-FX imaging platform (Vilber Lourmat, France).

## 2.9. Luciferase reporter assays

Type I IFN and IL1 $\beta$  production in supernatants of stimulated chicken HD11 cells or chBMDM was measured using luciferase-based Mx- or NF $\kappa$ B-reporter bioassays, respectively (Gyorffy et al., 2003; Schwarz et al., 2004). Briefly, CEC32-Mx or CEC32-NF $\kappa$ B cells were seeded at  $2.5 \times 10^5$  cells/well in 24-well plates and incubated at 41°C under 5% CO<sub>2</sub> overnight. The next day, cells were incubated for 6h with the diluted supernatants (1/10 of total volume) from stimulated HD11 or chBMDM cell cultures. Medium was removed and cells were washed twice with PBS. Cells were lysed using the Cell Culture Lysis Reagent (Promega, USA), according to the manufacturer's instructions, and luciferase activity was measured using the Luciferase assay reagent (Promega, USA) and a GloMax-Multi Detection System (Promega, USA). Data were expressed as IFN $\beta$  or IL1 $\beta$  activity (fold increase as compared to control group).

For NF $\kappa$ B activity measurement in HD11 cells, HD11-NF $\kappa$ B reporter cells were seeded at  $2.5 \times 10^5$  cells/well in 24-well dishes and incubated at 41°C under 5% CO<sub>2</sub> overnight. The next day, HD11-NF $\kappa$ B cells were incubated for 6h with the indicated stimuli and inhibitors at the appropriate concentration. Next, medium was removed and cells were treated as described for CEC32-Mx or CEC32-NF $\kappa$ B cells. Data are expressed as NF $\kappa$ B activity (fold increase relative to the control or mock group).

## 2.10. Phagocytosis and endocytosis assay



Phagocytosis or endocytosis (fluid-phase pinocytosis and receptor-mediated endocytosis) capacity of HD11 cells were evaluated by flow cytometry using pHrodo™ Green *E. coli* BioParticles® Conjugate or pHrodo™ Green Dextran (Invitrogen, USA), respectively. Briefly, HD11 cells were seeded in 48-well plates at  $2 \times 10^5$  cells/well and incubated at 41°C under 5% CO<sub>2</sub> overnight. Then, cells were pretreated with IFN $\alpha$  (50 ng/ml) for 16h (unless otherwise indicated). The next day, cells were gently washed with PBS then incubated with fresh incomplete RPMI 1640 containing pHrodo™ Green Dextran at 50  $\mu$ g/ml or with incomplete RPMI 1640 containing unopsonized pHrodo™ Green *E. coli* BioParticles® Conjugate at 333  $\mu$ g/ml for 30 min and 1h, respectively. Cells were washed then harvested with pre-warmed FACS buffer and subsequently analyzed by flow cytometry (BD FACS Calibur). Data were expressed as the percentage of pHrodo green<sup>+</sup> cell populations over total acquired events (50,000 cells).

## 2.11. Statistical analysis

Comparisons between two groups were performed using a two-tailed unpaired Student's t test. Multiple groups were compared using a one-way ANOVA analysis followed by a Tukey multiple comparison post-hoc test. Values for all measurements are expressed as mean  $\pm$  SEM.  $P < 0.05$  was considered statistically significant. Data are representative of at least two independent experiments unless otherwise indicated. Statistical analysis was performed using the GraphPad Prism 6.0 software (GraphPad Software, USA).

## 3. Results

### 3.1. IFN $\alpha$ and IFN $\beta$ elicit different intracellular responses in chicken primary macrophages

To gain first insights into the functional roles played by type I IFNs in chicken primary macrophages, we compared the expression profiles of various ISGs, IFN-regulatory transcription factors and the pro-inflammatory responses elicited by IFN $\alpha$  and IFN $\beta$ . At 2h (data not shown) and 6h following type I IFNs stimulation, chBMDM showed increased expression of the ISGs 2'-5' oligoadenylate synthetase (*OAS1*), myxovirus resistance protein (*MX1*), double-stranded RNA-activated protein kinase (*EIF2AK2*) and signal transducer and activator of transcription 1 (*STAT1*) as compared to the mock control group (**Figure 1A**). Among the ISGs and associated transcription factors, only the expression of interferon regulatory factor 7 (*IRF7*) proved to be more elevated after IFN $\beta$  stimulation at the tested time-points, while all other ISGs were more efficiently induced by IFN $\alpha$ . Yet, pro-inflammatory genes such as *IL1B*, *IFNB*, *IL6* and *IL8L2* were markedly up-regulated upon IFN $\beta$  treatment as compared to cells treated with IFN $\alpha$  (**Figure 1B**). Our data therefore demonstrated that, at least at early time-points, both type I IFNs play a role in triggering ISGs expression, but IFN $\beta$  has a more pronounced effect on pro-inflammatory cytokine gene expression in chBMDM.

In mammals, STAT1, mitogen-activated protein kinases (MAPKs) p38 and p44/42 (Erk1/2), PI3K/Akt, and NF $\kappa$ B pathways have been shown to be involved in type I IFN signalling (Hervas-Stubbs et al., 2011). We therefore performed western blot analyses to investigate whether some of these signalling pathways were triggered by type I IFNs in chBMDM. STAT1 expression was strongly induced from 6h to 24h upon exposure to IFN $\alpha$  as compared to the mock control group (**Figure 2A**). These results confirmed that the upregulation of STAT1 protein is correlated to the gene expression data (**Figure 1A**). Furthermore, IFN $\alpha$  stimulation markedly induced early phosphorylation and activation of STAT1 (pSTAT1), which lasted for up to 24h, as compared to the mock control group. In contrast to the situation

in cells stimulated with IFN $\alpha$ , STAT1 and pSTAT1 were only slightly induced by IFN $\beta$ , both displaying a modest, yet sustained induction at 24h post-stimulation (**Figure 2A**).

We also found that p38 MAPK, p44/42 MAPK and Akt were constitutively expressed in chBMDM (**Figure 2A**). The p38 MAPK phosphorylated form (pp38) was expressed from 30 min to 6h following stimulation with IFN $\alpha$  or IFN $\beta$ . Only low levels of pp38 were detected 24h after stimulation with both type I IFNs, suggesting that the p38 MAPK pathway is activated only at early time-points following stimulation. In contrast to the rapid and clear expression pattern observed for pp38, pp44/42 expression was weakly induced from 2h to 24h following stimulation with IFN $\alpha$  or IFN $\beta$ . This might suggest a minor (or indirect) role of these cytokines in the activation of this pathway in chBMDM. In regard to the PI3K/Akt pathway, pAkt expression was weakly induced by IFN $\alpha$ . However, stimulation with IFN $\beta$  markedly upregulated pAkt expression as early as 1h, with a quick decrease to mock control group levels after 2h. Therefore, the PI3K/Akt pathway is likely to be better activated by IFN $\beta$  as compared to IFN $\alpha$  in chBMDM. Finally, we investigated the role of type I IFNs in the expression of proteins involved in the activation of NF $\kappa$ B. Activation occurs via phosphorylation of I $\kappa$ B $\alpha$  at Ser32 and Ser36 followed by proteasome-mediated degradation that results in the release and nuclear translocation of active NF $\kappa$ B (Hayden and Ghosh, 2008). Although we could not detect the expression of I $\kappa$ B $\alpha$  in chBMDM using the present western blot protocol and commercially available antibodies (data not shown), we demonstrated that IFN $\beta$ , but not IFN $\alpha$ , induced a marked expression of pI $\kappa$ B $\alpha$  (Ser32/36) at 6h post-stimulation (**Figure 2A**).

To better characterize the potential activation NF $\kappa$ B by type I IFNs, we used an HD11-NF $\kappa$ B luciferase reporter cell line. Cells were stimulated for 6h with IFN $\alpha$  or IFN $\beta$ , and LPS treatment was included as a positive control. As expected, and consistent with its incapacity to directly induce a pro-inflammatory profile, the NF $\kappa$ B pathway was not triggered by IFN $\alpha$ . In

contrast, it was strongly activated by IFN $\beta$  as compared to the mock control group (**Figure 2B**), which is in line with its ability to induce phosphorylation of I $\kappa$ B $\alpha$  at 6h (**Figure 2A**). In addition, we observed that pharmacological inhibition of the intracellular signalling pathways p38 MAPK, PI3K/Akt and TBK1/IKK $\epsilon$  partially reduced the activation of NF $\kappa$ B induced by IFN $\beta$  (**Figure 2B**). The 7-AAD staining protocol revealed that none of the inhibitors were found to be cytotoxic (necrotic cell death) at the concentrations used (data not shown). These data suggest that the signalling pathways studied are likely to be involved in the activation of NF $\kappa$ B following stimulation with IFN $\beta$ .

Taken together, our results revealed that IFN $\alpha$  and IFN $\beta$  play different roles in the induction of chicken macrophage intracellular signalling pathways upstream of the transcriptional regulation of ISGs or pro-inflammatory genes.

### **3.2. IFN $\alpha$ elicits similar response patterns in a chicken macrophage cell line and in lung macrophages**

IFN $\alpha$  is the best studied type I IFN in birds (Giotis et al., 2016; Goossens et al., 2013; Roll et al., 2017; Santhakumar et al., 2017). We therefore complemented our findings by assessing the impact of IFN $\alpha$  in the well-established chicken macrophage cell line HD11 (Beug et al., 1979). Incubation of HD11 cells with IFN $\alpha$  for 6h led to a marked increase in the expression of the ISGs *OAS1*, *MX1* and *EIF2AK2* as compared to the mock control group (**Figure 3A**). The interferon-regulated transcription factors *IRF7* and *STAT1* likewise showed a significant increase in their transcriptional expression (3 and 7 fold, respectively) (**Figure 3A**). IFN $\alpha$  stimulation did not alter *IL1B*, *NOS2*, *IFNA* and *IFNB* gene expression and nitric oxide (NO) production in HD11 supernatants (**Figure 3B**). Furthermore, we confirmed by flow cytometry

analysis using Annexin V and 7-AAD staining that IFN $\alpha$  was not cytotoxic (late apoptosis/necrosis) to HD11 cells after 6h or 16h of stimulation (**Figure 3C**).

To better improve our knowledge on the responses elicited by IFN $\alpha$  in chicken macrophages, gene expression data for HD11 cells were compared to those obtained from chicken lung macrophages at a matching time-point (6h). Except for *OAS1*, ISGs expression in lung macrophages was significantly higher (307%, 154%, 300% and 100% for *EIF2AK2*, *MX1*, *IRF7* and *STAT1*, respectively) than those found in HD11 cells (**Figure 3D**). Interestingly, the baseline Ct values for the aforementioned ISGs (except for *OAS1*) are very similar between HD11 cells and lung macrophages (**Supplementary Table 1**). Similarly to HD11 cells, no increase in pro-inflammatory gene expression was observed (data not shown).

Consequently, the HD11 cell line was used in most experiments of the present study, because of its easy accessibility, handling, and maintenance. Nevertheless, the most relevant findings were further confirmed using chicken primary macrophages.

### **3.3. IFN $\alpha$ priming potentiates the pro-inflammatory response to *E. coli* LPS in chicken macrophages**

As demonstrated in chicken lung macrophages and in a cell line, IFN $\alpha$  strongly induced ISGs but a negligible pro-inflammatory gene expression profile. Indeed, IFN $\alpha$  has been used in priming strategies to assess the impact of type I IFNs in cellular responses to cytokines, pathogen-associated molecular patterns (PAMPs) or pathogens in chickens and mammalian species (Doughty et al., 2001; Jiang et al., 2011; Pei et al., 2001; Sharif et al., 2004). We therefore asked whether a type I IFN enriched environment, likely to be encountered during viral infections, could modulate the chicken macrophage inflammatory response to *E. coli* LPS. We stimulated HD11 cells by incubating them with IFN $\alpha$  for 16h (priming time).

Shorter and longer exposition times were tested and showed to be less effective in inducing a non-cytotoxic priming activity in this macrophage cell line (data not shown). HD11 cells were subsequently stimulated for 6h with *E. coli* LPS (**Figure 4A**), a time point where gene expression or NO production were peaking or produced consistently reproducible data.

We observed that IFN $\alpha$  priming followed by LPS stimulation (IFN $\alpha$  prm + LPS) markedly upregulated *IL1B*, *NOS2* and *IFNB* gene expression, concomitant with NO and ROS production, as compared to the group treated with LPS alone (**Figure 4B and 4C**). The same stimulating effect was observed for the expression of the genes of interest at 2h (data not shown). As expected, IFN $\alpha$  priming (IFN $\alpha$  prm) alone and IFN $\alpha$  stimulation (6h) had no effect on pro-inflammatory gene expression and were ineffective in promoting ROS or NO production (**Figure 4B and 4C**). We next used a bioassay to verify whether the *IFNB* upregulation was associated to IFN $\beta$  production by chicken macrophages. Under all treatment conditions tested, *IFNA* gene expression was assed using different primer pairs (data not shown) and was found to be never induced in HD11 cells, suggesting that the type I IFN bioactivity determined in the bioassay would largely rest on IFN $\beta$  production. Corroborating the gene expression data, we observed that more IFN $\beta$  was produced following LPS stimulation when HD11 cells were previously primed with IFN $\alpha$  (**Figure 5A**). Although IFN $\alpha$  priming potentiated the pro-inflammatory response to LPS, no additional cytotoxic effect was observed when both molecules were added to the cells as compared to the group receiving LPS alone (**Figure 5B**).

In addition, we evaluated the impact of IFN $\alpha$  and/or LPS on the expression of co-stimulation markers by HD11 cells. Only LPS was able to upregulate CD40 expression, whereas IFN $\alpha$  priming had no potentiating effect (**Figure 5C**). However, MHC II expression was increased by IFN $\alpha$  priming (16h), but not by IFN $\alpha$  stimulation (6h). The addition of LPS did not

increase MHC II expression in priming or mock conditions at the same time-points (**Figure 5C**).

We confirmed these results by using chBMDM and applying the same experimental approach. *IL1B*, *NOS2*, *IFNB* expression, and NO, IFN $\beta$  and IL-1 $\beta$  production (as assessed using the CEC32-NF $\kappa$ B reporter cell line), were all upregulated when chBMDM were primed with IFN $\alpha$  for 16h and then challenged with LPS for 2h (data not shown) or 6h (**Supplementary Figure 1A and 1B**). Interestingly, we observed that IFN $\alpha$  stimulation induced *IL1B* and *NOS2* expression concomitant with NO production, which rapidly decreased to control group levels. Notably, neither gene expression nor NO production was observed in the IFN $\alpha$  primed group (16h), therefore excluding any potential cumulative effects on the priming approach. Strikingly, the bioassay revealed that IFN $\beta$  was also produced in the IFN $\alpha$  primed group. However, HD11 cells were washed with PBS after the priming to avoid any contamination of IFN $\alpha$  in the culture supernatant, therefore suggesting a sustained secretion of IFN $\beta$  by these cells (**Supplementary Figure 1B**).

In conclusion, IFN $\alpha$  priming favours the development of an increased pro-inflammatory response to *E. coli* LPS in chicken macrophages without entailing cytotoxic effects. Moreover, IFN $\alpha$  increases MHC II expression on the HD11 cell line, suggesting an increased antigen presentation potential.

### **3.4. IFN $\beta$ mediates the increased pro-inflammatory response to *E. coli* LPS in chicken macrophages following IFN $\alpha$ priming**

We observed that IFN $\beta$  production induced by LPS was strongly enhanced by IFN $\alpha$  priming in chicken macrophages. Previous studies have demonstrated that IFN $\beta$  is involved in different pro-inflammatory processes in mammals and chickens (de Weerd and Nguyen,

2012; Hervás-Stubbs et al., 2011; Santhakumar et al., 2017). We therefore speculated that IFN $\beta$  could be involved in the onset of a pro-inflammatory state induced by IFN $\alpha$  priming. To test this, HD11 cells were treated as described earlier and a rabbit anti-chicken IFN $\beta$ -antiserum was added along with LPS in order to neutralize IFN $\beta$  released into the medium. As shown in **Figure 6A**, in the group primed with IFN $\alpha$  and subsequently challenged with LPS for 2h (data not shown) and 6h, the addition of IFN $\beta$ -antiserum (right bars) virtually abrogated the potentiation effects of the priming protocol on *IL1B*, *NOS2* and *IFNB* expression, and on NO and IFN $\beta$  production. Importantly, IFN $\beta$ -antiserum did not affect the pro-inflammatory response induced by LPS alone (**Figure 6A**), and IFN $\beta$ -antiserum was not cytotoxic for chicken macrophages (**Figure 6B**).

The same experiments were also performed using chBMDM, in which IFN $\beta$ -antiserum addition similarly abrogated the potentiating effect of IFN $\alpha$  priming on the LPS-induced pro-inflammatory response (**Supplementary Figure 2**). To corroborate our findings, we also incubated chBMDM with LPS and IFN $\beta$ . We observed that, when added together, LPS and IFN $\beta$  mimicked the potentiating pro-inflammatory effect induced by IFN $\alpha$  priming (**Figure 6C**).

Our data thus suggest that IFN $\beta$  is a key mediator of the increased pro-inflammatory response to LPS observed in chicken macrophages previously primed with IFN $\alpha$ .

### **3.5. IFN $\alpha$ priming potentiates chicken macrophage pro-inflammatory responses to APEC infection paralleled by an increased phagocytosis capacity**



Since IFN $\alpha$  priming potentiates the chicken macrophage pro-inflammatory response to *E. coli* LPS, we next explored whether IFN $\alpha$  priming modulates the macrophage response to avian pathogenic *E. coli* (APEC) infection. HD11 cells were primed with IFN $\alpha$  for 16h then infected with the highly adhesive/invasive APEC strain BEN2908 at an MOI of 10 for 6h. Cells primed with IFN $\alpha$  and infected with the APEC strain (IFN $\alpha$  prm + APEC) showed an up-regulation of *IFNB* expression, concomitant with an increased NO and IFN $\beta$  production, when compared to the non-primed BEN2908-infected group (APEC) (**Figure 7A**). *IL1B* was also up-regulated in the primed group, but no statistical difference was seen compared to the non-primed APEC group (**Figure 7A**).

In addition, we counted intracellular bacteria at 1h and 2h 30 min post infection in order to assess the number of adhered and intracellular bacteria, respectively. Bacterial adhesion was not affected by IFN $\alpha$  priming (**Figure 7B**) whereas the number of viable intracellular bacteria was significantly increased when macrophages were primed with IFN $\alpha$  (**Figure 7C**).

We next asked whether the increased intracellular bacterial load could be mediated by an IFN $\alpha$ -dependent enhancement of the endocytic or phagocytic capacity of HD11 cells. Subsequent to several pilot experiments to identify the best time-points and reagents concentrations to be used in these experiments (data not shown), endocytosis (fluid-phase pinocytosis and receptor-mediated endocytosis) and phagocytosis were evaluated by flow cytometry using fluorescent particles. We found that IFN $\alpha$  priming did not impact endocytosis (**Figure 7D**) but it was able to significantly enhance the phagocytosis capacity of chicken macrophages (20% increase) (**Figure 7E**).

The increase in intracellular bacterial uptake observed in HD11 cells following IFN $\alpha$  priming was confirmed in experiments with chicken lung macrophages using a BEN2908 strain expressing the GFP (**Figure 7F**). In addition, intracellular bacterial fluorescence was assessed

by flow cytometry and found to be higher in lung macrophages primed with IFN $\alpha$  (**Figure 7G**).

Altogether, these results evidenced that IFN $\alpha$  priming potentiates the pro-inflammatory response to APEC infection in chicken macrophages paralleled by an increased phagocytic activity, without detectable bactericidal consequences.

#### 4. Discussion

Macrophage functions such as pathogen recognition, phagocytosis and cytokine expression have been shown to be greatly impacted by type I IFNs in mammalian systems (Lee et al., 2015; Nakamura et al., 2011; Shahangian et al., 2009; Shepardson et al., 2016). In chickens, although extensive work have been done to understand type I IFN biology and their inhibitory effects on virus replication (Giotis et al., 2016; Jiang et al., 2011; Mo et al., 2001; Pei et al., 2001; Roll et al., 2017), many aspects of the type I IFN response remain unexplored, notably in regard to macrophages and their inflammatory response. Our data revealed that for chicken primary macrophages IFN $\alpha$  was a more potent inducer of ISGs expression (*OAS*, *MX1*, *PKR* and *STAT1*) when compared to IFN $\beta$  at 6h post stimulation. In contrast, at the same time point post stimulation, IFN $\beta$  proofed to be a better inducer of pro-inflammatory cytokine gene expression (*IL1B*, *IFNB*, *IL6* and *IL8L2*). A previous study using the DF-1 chicken fibroblast cell line showed that IFN $\alpha$  stimulation entails a strong antiviral profile, mainly through a marked upregulation of ISGs associated to robust antiviral activity (Qu et al., 2013). In contrast, IFN $\beta$  appears to rather drive an immune modulatory response. Our data obtained with chicken macrophages are in good agreement with this observation. Several hypothesis have been made to explain these differential effects, including putative different affinities of type I IFNs to the subunits of their cognate receptor (IFNAR1 and IFNAR2) (Santhakumar et

al., 2017). However, type I IFN signalling and the resulting gene expression patterns are likely to be different between chicken macrophages and fibroblasts, due to the different biological functions of these cells.

The distinct activity profiles observed for type I IFNs in the present study might be explained by differences in the activation of intracellular signalling pathways. In mammals, STAT1 is an important mediator of the JAK/STAT pathway in type I IFNs signalling, leading to the transcription of ISGs (Hervas-Stubbs et al., 2011). In chicken macrophages, STAT1 phosphorylation was only weakly induced by IFN $\beta$ , but remained on a steady level throughout the stimulation period. In contrast, IFN $\alpha$  rapidly induced STAT1 phosphorylation. We speculate that the differences in ISGs expression induced by the two type I IFNs is directly linked to differential STAT1 pathway activation. Conversely, the p38 MAPK pathway exhibited the same activation kinetics in response to both IFN $\alpha$  and IFN $\beta$ . The role of this signalling pathway for the induction of ISGs and other genes downstream of the type I IFN receptor IFNAR has been demonstrated in mice (Li et al., 2004), but remains unclear in birds.

Although 6h and 16h stimulation with IFN $\alpha$  alone was not found to strongly induce transcriptional expression of *IL1B*, *NOS2*, and *IFNB* in HD11 cells and primary macrophages, it potentiated the pro-inflammatory response to *E. coli* LPS, APEC LPS (data not shown) and APEC infection. We hypothesize that IFN $\alpha$  may prime or modify intracellular events in the macrophages, such as increasing the expression, production and/or activation of so far unrecognized transcription factors, thereby promoting the IFN $\beta$  over-production observed after challenge with bacterial molecular patterns. In mammals, type I IFNs act through JAK/STAT, CRB, PI3K/Akt, NF $\kappa$ B and MAPK signalling pathways (Hervas-Stubbs et al., 2011), which are still largely unexplored in chickens due to the lack of species-specific pharmacological inhibitors and antibodies. One study has previously demonstrated that

chicken IFN $\alpha$  and IFN $\beta$  promoters share binding regions for transcription factors of the IRF family, and that the IFN $\beta$  promoter has an additional NF $\kappa$ B binding site (Sick et al., 1998). We demonstrated here that only *IFNB* gene expression was induced after bacterial challenge, in agreement with previously published data (Barjesteh et al., 2014). IFN $\beta$  is known to induce NF $\kappa$ B activation (Hervas-Stubbs et al., 2011). We therefore assume that the NF $\kappa$ B pathway may be involved in IFN $\beta$  production induced by LPS and in the inflammatory responses elicited by IFN $\beta$ . In fact, when we compared the effects of IFN $\alpha$  and IFN $\beta$  using the HD11-NF $\kappa$ B reporter cell line and by assessing the phosphorylation of I $\kappa$ B $\alpha$ , we observed that IFN $\alpha$  did not activate this pathway, contrary to what was observed for IFN $\beta$ . On the other hand, in chicken primary macrophages, IFN $\alpha$  stimulation led to increased *IL1B* and *NOS2* gene expression, all of which have been shown to be induced by the NF $\kappa$ B pathway upon LPS stimulation in mammals and chickens (Aktan, 2004; Contassot et al., 2012; He and Kogut, 2003; Weining et al., 1998). This suggests that the regulation of the NF $\kappa$ B pathway in an immortalized cell line and in primary macrophages seems to be different.

NF $\kappa$ B activation by IFN $\beta$  in chicken macrophages is partially dependent on the p38 MAPK, PI3K/Akt and/or TBK1/IKK $\epsilon$  signalling pathways. To our knowledge, this is the first time that such a mechanism of action has been described for a type I IFN in chickens. Since pharmacological inhibition of these pathways did not completely abrogate NF $\kappa$ B activation in the HD11 cell line, other pathways involved in this activation remain to be identified. In addition, IFN $\beta$  induced the expression of *IRF7* and could therefore potentially activate this transcription factor. Since both NF $\kappa$ B and IRF7 may bind to specific binding motifs present in the IFN $\beta$  promoter (Sick et al., 1998), we assume that IFN $\beta$  might be able to enhance its own expression through an amplification loop involving the aforementioned signalling pathways. Bacterial LPS activates Toll-like receptor 4 (TLR4) and triggers intracellular signalling pathways leading to NF $\kappa$ B activation via Myd88-dependent or the Myd88-independent

TRIF/TRAM-dependent pathways in mammals (Takeda and Akira, 2007). It is well established in mammals that the TRIF/TRAM-dependent pathway also activates IRF3, which results in IFN $\beta$  induction (Kawai and Akira, 2010). Previous studies have demonstrated that LPS-induced IFN $\beta$  is crucial for LPS-dependent NO production (Vadiveloo et al., 2000), pro-inflammatory cytokine and chemokine expression (Thomas et al., 2006), and LPS-derived ISGs expression (Sheikh et al., 2014) in murine macrophages. In chickens, little is known regarding the contribution of IFN $\beta$  to LPS-induced inflammatory responses. Moreover, an orthologue of the *Ticam2* gene (encoding TRAM) appears to be missing from the chicken genome, suggesting that TLR4 signalling through TRIF/TRAM might not be functional or ineffective in LPS-stimulated chicken cells (Kestra and van Putten, 2008). Nevertheless, our findings demonstrated that *E. coli* LPS or APEC are able to induce IFN $\beta$  production in chicken macrophages, corroborating previous data obtained with the MQ-NCSU chicken macrophage cell line (Barjesteh et al., 2014).

Although all the molecular events involved in IFN $\alpha$ -induced macrophage reprogramming could not be identified, we demonstrated that the IFN $\alpha$ -activated STAT1 and p38 MAPK signalling pathways are likely to play a role in rendering macrophages more prone to mount an inflammatory response (via IFN $\beta$  production) after bacterial challenge. Based on our data, we assume that the cross-talk between signalling pathways downstream of TLR4 (e.g. NF $\kappa$ B, p38 MAPK and IRF7) and IFNAR leads to the potentiated production of IFN $\beta$ , which in turn activates NF $\kappa$ B via p38 MAPK, PI3K/Akt, TBK1/IKK $\epsilon$ , and eventually other unidentified pathways, ultimately resulting in the potentiated production of pro-inflammatory mediators.

Previous studies demonstrated that IFN $\alpha$  stimulation increases mouse peritoneal macrophage phagocytosis and bacterial adhesion through potential modifications of membrane surface receptors such as Fc or C3b receptors (Rollag et al., 1984). In the present study, we observed that bacterial adhesion remained unchanged upon IFN $\alpha$  stimulation. Therefore, we surmise

that specific and non-specific bacterial receptors (e.g. scavenger receptors) may not account for the enhancement in macrophage phagocytosis. In addition, Fc receptors do not appear to be involved in this phenomenon since neither bacteria nor bio-particles were opsonized. We thus assume that IFN $\alpha$  stimulation is able to induce unrecognized metabolic modifications within chicken macrophages that led to an increased phagocytic activity. This mechanism is likely to be IFN $\beta$ -independent since IFN $\beta$ -antiserum treatment did not reduce phagocytic activity of IFN $\alpha$ -primed macrophages (data not shown).

A recent study demonstrated that a preceding LPAIV H9N2 infection increased innate immunity-related gene expression in response to LPS challenge in the HD11 cell line (Qi et al., 2016). Here we showed that a type I IFN enriched environment, likely to be present in mucosal surfaces (respiratory and intestinal tract) infected with LPAIV, was sufficient to reproduce this phenomenon in chicken macrophages. However, in the LPAIV infection-LPS challenge system, *IFNA* and *TLR4* expression was found to be upregulated, a phenomenon that was not observed in the present work, suggesting that these responses could be type I IFN-independent. It would be challenging to test whether this pro-inflammatory response might also occur *in vivo* in an LPAIV-APEC superinfection model. Yet, previous studies indicated that LPAIV infection may pave the way for clinical colibacillosis in poultry (Bano et al., 2003; Mosleh et al., 2017; Umar et al., 2018). Even though type I IFNs are produced during viral infections, including low pathogenic avian influenza, the pathogenesis of viral/bacterial co-infections may be very different depending on the viral pathogen or strain. For example, during infectious bronchitis virus (IBV) infection, the type I IFN response remained unchanged between co-infected and *E. coli* infected groups, suggesting that a type I IFN response was not involved in the exacerbation of colibacillosis (Ariaans et al., 2008).

Altogether, our data provide the first evidence for the role of type I IFNs in modifying chicken macrophage homeostasis, which may translate into a prominent pro-inflammatory

phenotype mediated by IFN $\beta$  when these cells encounter bacteria. Our findings point to an eminent role of this mechanism in the pathogenesis of viral/bacterial co-infections in the chicken that warrants further investigation by an in-depth analysis of the innate immune response in experimentally (co-)infected animals.

## Acknowledgments

We thank the animal experimental unit PFIE (Plateforme d'Infectiologie Expérimentale, Centre INRA Val de Loire, Nouzilly, France) and The National Avian Research Facility, Roslin Institute, UK, for providing the animals used for the isolation of primary macrophages. This study received financial support from the European program ERA-NET ANIHW (Animal Health and Welfare) through funding of the MICHIC project (Understanding mucosal immunology and co-infections in the chicken to drive vaccine strategies) and from The Roslin Institute Strategic Grant funding from the BBSRC (BB/P013740/1). DG was supported by a PhD fellowship from the Région Centre Val de Loire, France.

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## Figure Legends

### Figure 1. IFN $\alpha$ and IFN $\beta$ elicit different responses in chicken primary macrophages.

chBMDM were stimulated with IFN $\alpha$  (50 ng/ml) or IFN $\beta$  (50 ng/ml) for 6h before qRT-PCR analysis. (A) *OAS/OAS1*, *Mx/MX1*, *PKR/EIF2AK2*, *STAT1/STAT1* and *IRF7/IRF7* gene expression. (B) *IL-1 $\beta$ /IL1B*, *IFN $\beta$ /IFNB*, *IL-6/IL6*, *CXCLi2/IL8L2* gene expression. qRT-PCR data are expressed as relative normalized expression (as compared to mock control

group). Values are  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  or \*\*\*\* $P < 0.0001$  when compared to mock control group. # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  or #### $P < 0.0001$  when compared to IFN $\alpha$  group. Data are representative of two independent experiments performed in triplicates.

**Figure 2. IFN $\alpha$  and IFN $\beta$  differently modulate chicken primary macrophages signalling pathways.** (A) chBMDM were stimulated with IFN $\alpha$  (50 ng/ml) or IFN $\beta$  (50 ng/ml) for 30 min, 1h, 2h, 6h or 24h, then western blot analysis was performed on cell lysates. Representative immunoblotting revelations of STAT1, pSTAT1, p38, ppP38, p44/42, pp44/42, Akt, pAkt and pI $\kappa$ B $\alpha$  are shown. Protein molecular weight is indicated with black arrows. GAPDH was used as loading control. (B) HD11-NF $\kappa$ B reporter cells were incubated with IFN $\alpha$  (50 ng/ml), LPS (10 ng/ml) or IFN $\beta$  (50 ng/ml) alone or in combination with pharmacological inhibitors (SB-203580 at 10  $\mu$ M, Wortmannin at 2  $\mu$ M, BX795 at 2  $\mu$ M and BAY11-7082 at 10  $\mu$ M) for 6h then luciferase activity was measured. Data are expressed as fold increase in NF $\kappa$ B activity, as compared to control groups, and values are  $\pm$  SEM. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  or \*\*\*\* $P < 0.0001$  when compared to mock control group. # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  or #### $P < 0.0001$  when compared to the IFN $\alpha$  group (left graph) or the IFN $\beta$  group (right graph). Data are representative of two (A) or three (B) independent experiments performed in triplicates.

**Figure 3. IFN $\alpha$  elicits similar response patterns in a chicken macrophage cell line and in lung macrophages.** HD11 cells (A, B, and C) or macrophages from chicken lungs (D) were stimulated with IFN $\alpha$  (50 ng/ml) for 6h before qRT-PCR analysis, nitric oxide (NO) dosage or flow cytometry analysis for cell viability. (A) PKR/*EIF2AK2*, Mx/*MX1*, OAS/*OAS1*,

IRF7/*IRF7* and STAT1/*STAT1* gene expression, (B) IL-1 $\beta$ /*IL1B*, IFN $\alpha$ /*IFNA*, IFN $\beta$ /*IFNB*, iNOS/*NOS2* gene expression (left Y axis) and NO production (right Y axis). (C) Representative dot plot of HD11 double positive population for Annexin V and 7AAD, canonical markers of cell death, and histogram showing the percentage of double positive populations as compared to mock control group. (D) PKR/*EIF2AK2*, Mx/*MX1*, OAS/*OAS1*, IRF7/*IRF7* and STAT1/*STAT1* gene expression. Values are  $\pm$  SEM. qRT-PCR data are expressed as relative normalized expression (as compared to mock control group). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 or \*\*\*\*P < 0.0001 when compared to mock control group. Data are representative of two (C and D) or three (A and B) independent experiments performed in duplicates (A, B and C) or triplicates (D).

**Figure 4. IFN $\alpha$  priming potentiates the pro-inflammatory response to *E. coli* LPS in chicken macrophages.** (A) Experimental protocol: HD11 cells were primed with IFN $\alpha$  (50 ng/ml) or mock treatments for 16h. The medium was removed and cells were washed before receiving LPS (10 ng/ml), IFN $\alpha$  (50 ng/ml) or mock stimulation for 6h. (B) IL-1 $\beta$ /*IL1B*, iNOS/*NOS2*, IFN $\alpha$ /*IFNA*, IFN $\beta$ /*IFNB* gene expression and NO production. qRT-PCR data are expressed as relative normalized expression (as compared to mock control group). (C) Reactive Oxygen Species (ROS) production measured by flow cytometry using a fluorescent probe. Data are expressed as Mean Fluorescent Intensity (MFI). All values are  $\pm$  SEM. \*\*\*P < 0.001 or \*\*\*\*P < 0.0001 when compared to mock control group. ###P < 0.001 or #####P < 0.0001 when compared to LPS group. Data are representative of four (B) or two (C) independent experiments performed in triplicates.

**Figure 5. IFN $\alpha$  priming boosts MHC class II expression and IFN $\beta$  production in response to *E. coli* LPS stimulation without exerting any measurable cytotoxic effect on chicken macrophages.** (A) IFN $\beta$  production was quantified in HD11 supernatants through a bioassay using a CEC32-Mx luciferase reporter cell-line. Data are expressed as fold increase in IFN $\beta$  activity as compared to control groups. (B) Cell viability was assessed by flow cytometry using 7-AAD. Data are expressed as the percentage of dead cells (7AAD positive). (C) CD40 or MHC class II expression was analysed by flow cytometry. Data are expressed as Mean Fluorescence Intensity (MFI) from FITC<sup>+</sup> cells. All values are  $\pm$  SEM. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 or \*\*\*\*P < 0.0001 when compared to mock control group. #####P < 0.0001 when compared to LPS group. Data are representative of two independent experiments performed in triplicates.

**Figure 6. IFN $\beta$  mediates the increased pro-inflammatory response to *E. coli* LPS in chicken macrophages following IFN $\alpha$  priming.** HD11 cells (A and B) or chBMDM (C) were primed with IFN $\alpha$  (50 ng/ml) or mock treatments for 16h, then stimulated for 6h with LPS (10 ng/ml) and/or IFN $\beta$ -antiserum (17.5  $\mu$ g/ml), and/or IFN $\beta$  (50 ng/ml) or IFN $\alpha$  (50 ng/ml). IL-1 $\beta$ /*IL1B*, IFN $\beta$ /*IFNB*, iNOS/*NOS2* gene expression and NO production in HD11 (A) and in chBMDM (C). qRT-PCR data are expressed as relative normalized expression (as compared to mock control group). (B) Cell viability was assessed by flow cytometry using 7-AAD. Data are expressed as the percentage of dead cells (7AAD positive). Values are  $\pm$  SEM. \*P < 0.05, \*\*P < 0.01 or \*\*\*\*P < 0.0001 when compared to mock control group. #P < 0.05 or #####P < 0.0001 when compared to LPS group. Data are representative of two (B and C) or three (A) independent experiments performed in duplicates.



**Figure 7. IFN $\alpha$  priming potentiates the chicken macrophage pro-inflammatory response to APEC infection paralleled by an increased phagocytosis capacity.** HD11 cells (**A**, **B**, **C**, **D** and **E**) or chicken lung macrophages (**F** and **G**) were primed with IFN $\alpha$  (50 ng/ml) or mock treatments for 16h, then cells were infected with 10 MOI of APEC BEN2908 or BEN2908 GFP respectively, for 1h (adhesion assessment). Next, cells were treated with gentamycin to kill extracellular bacteria for 1h 30 min (intracellular bacterial load assessment) and incubated for up to 6h (for transcriptomic analysis). (**A**) IL-1 $\beta$ /*IL1B* and IFN $\beta$ /*IFNB* gene expression, as assessed by qRT-PCR, and NO and IFN $\beta$  production as assessed through nitrite dosage and CEC32-Mx bioassay, respectively. The number of adhered bacteria (**B**) and intracellular bacteria (**C** and **F**) was evaluated through colony-forming units (CFU) counts. Endocytosis (**D**) and phagocytosis (**E**) capacities were evaluated by flow cytometry. Cells were pre-treated with IFN $\alpha$  (50 ng/ml) or mock treatments for 16h, and incubated for 30 min with pHrodo<sup>TM</sup> Green dextran (endocytosis) or for 1h with pHrodo<sup>TM</sup> Green *E. coli* BioParticles<sup>®</sup> Conjugate (phagocytosis) at 50  $\mu$ g/ml and 333 $\mu$ g/ml, respectively. Data are expressed as the percentage of pHrodo Green<sup>+</sup> cells. (**G**) The fluorescence from intracellular GFP-expressing bacteria was evaluated by flow cytometry and is expressed as Mean Fluorescence Intensity (MFI) from GFP<sup>+</sup> cells. Values are  $\pm$  SEM. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 or \*\*\*\*P < 0.0001 when compared to mock control group. #####P < 0.0001 when compared to APEC group. Data are representative of two (F and G) or three (A, B, C, D and E) independent experiments performed in triplicates.

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Target genes	Forward primers 5'-3'	Reverse primers 5'-3'
<i>B2M</i> ( $\beta$ 2M)	CGTCCTCAACTGCTTCGCG	TCTCGTGCTCCACCTTGC
<i>HPRT</i> (HPRT)	TGGTGGGGATGACCTCTCAA	GGCCGATATCCCACACTTCG
<i>GAPDH</i> (GAPDH)	GTCCTCTCTGGCAAAGTCCAAG	CCACAACATACTCAGCACCTGC
<i>EIF2AK2</i> (PKR)	GGGACATGATTGAGCCAAAGCAAGA	GAGCGTGGGGGTCTCCGGTA
<i>MX1</i> (M <sub>x</sub> )	ACGTCCCAGACCTGACACTA	TTTAGTGAGGACCCCAAGCG
<i>OAS1</i> (OAS)	CTTCGGAGTCAGCATCACCA	TCCTGAATCACCTGCCCCAG
<i>IRF7</i> (IRF7)	TGCCTCAGGCGTCCCAATG	TGTGTGCCACAGGGTTGGC

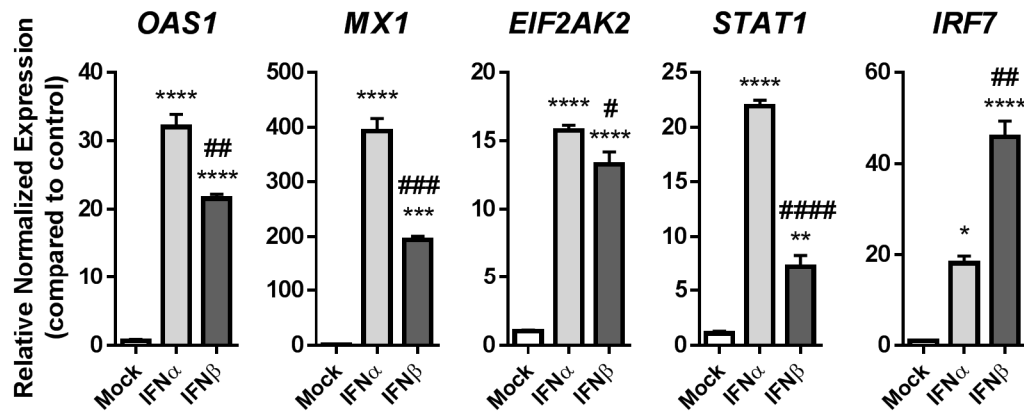
976



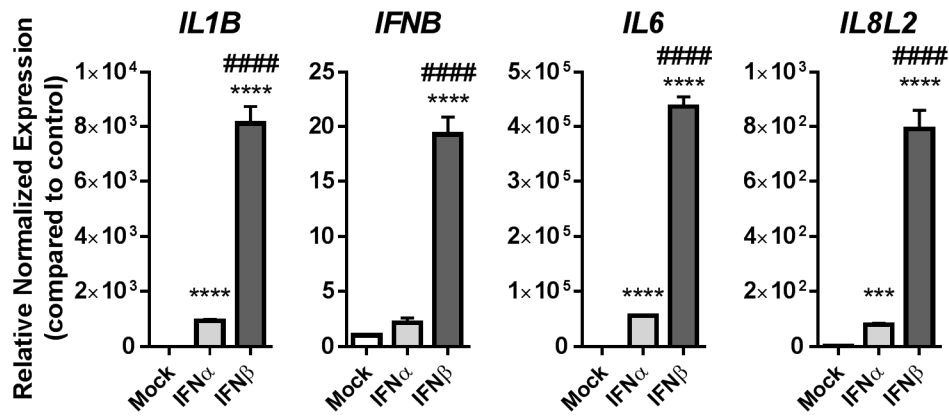
<i>STAT1</i> (STAT1)	AAGCAAACGTAATCTTCAGGATAAC	TTTCTCTCCTCTTTCAGACAGTTG
<i>IL1B</i> (IL1 $\beta$ )	AGGCTCAACATTGCGCTGTA	CTTGTAGCCCTTGATGCCCA
<i>IFNA</i> (IFN $\alpha$ )	CAACGACACCATCCTGGACA	GGGCTGCTGAGGATTTTGAA
<i>IFNB</i> (IFN $\beta$ )	TCCTGCAACCATCTTCGTCA	CACGTCTTGTTGTGGGCAAG
<i>NOS2</i> (iNOS)	CCACCAGGAGATGTTGAACTATGTC	CCAGATGTGTGTTTTCCATGCA
<i>IL6</i> (IL6)	GCTTCGACGAGGAGAAATGC	GCCAGGTGCTTTGTGCTGTA
<i>IL8L2</i> (CXCLi2)	CTGCGGTGCCAGTGCATTAG	AGCACACCTCTCTTCCATCC

**Table 1.** Primer pairs used in the present study for qRT-PCR analysis

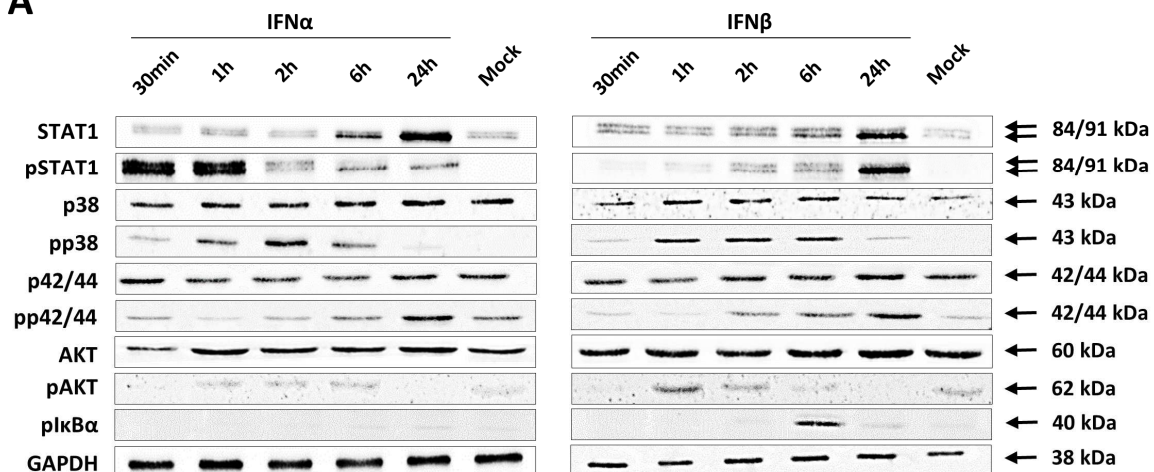
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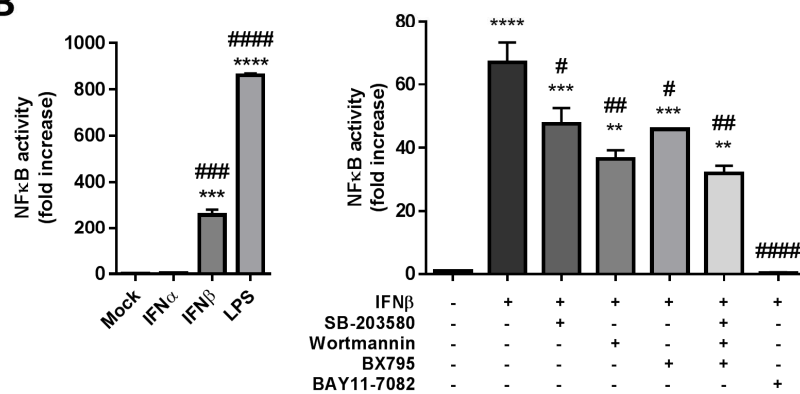
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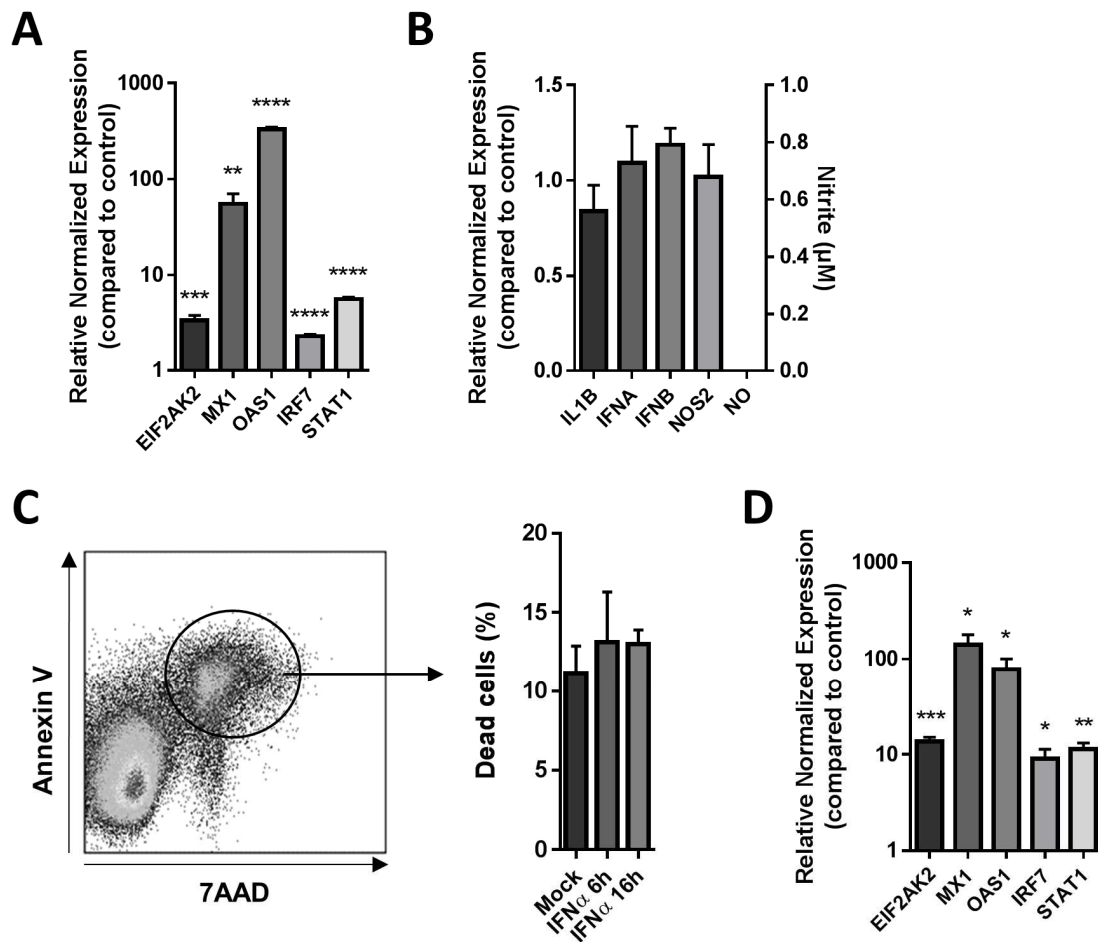


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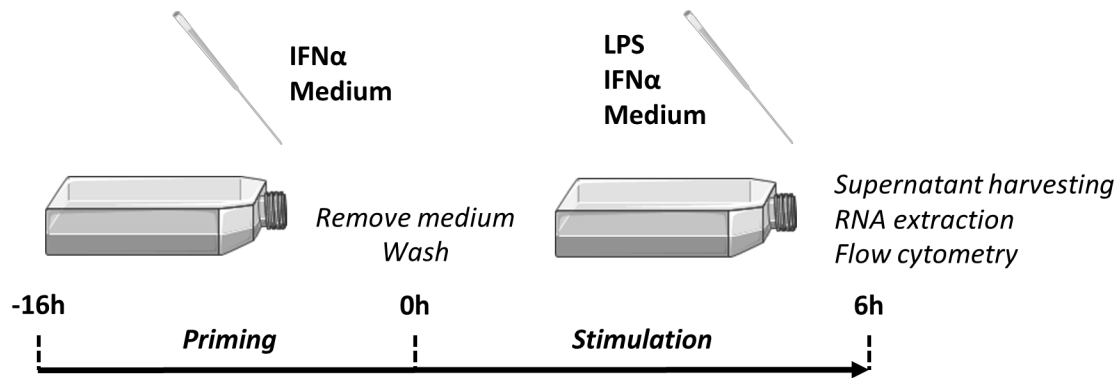


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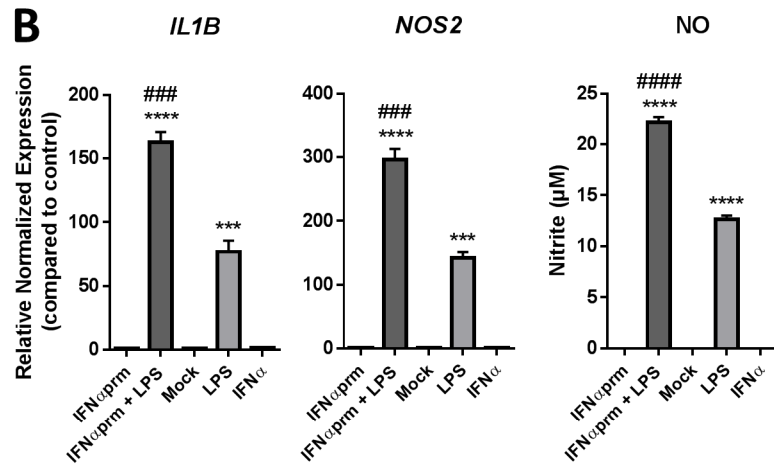




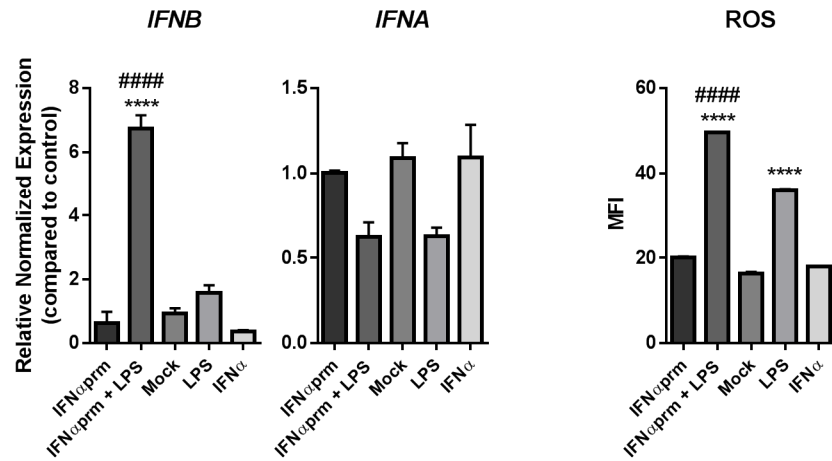
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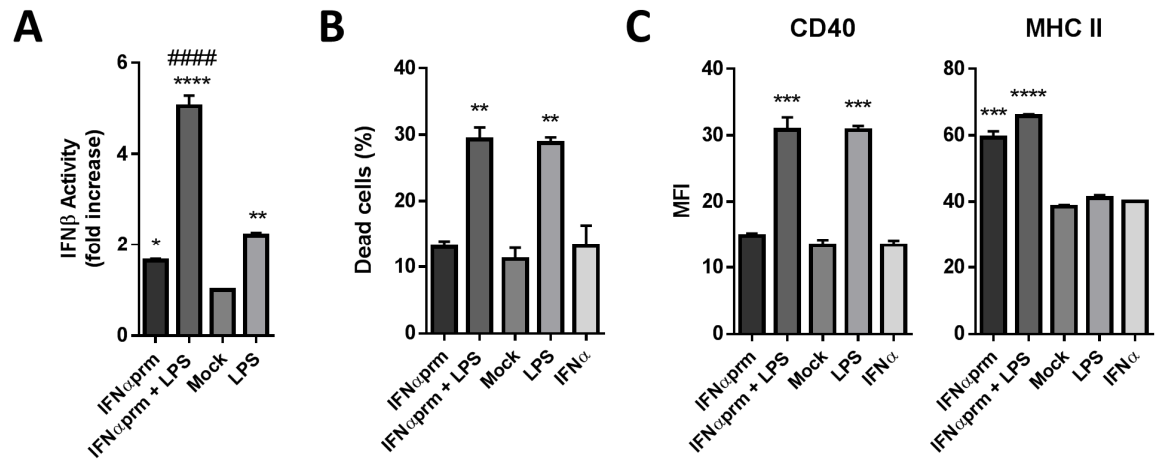


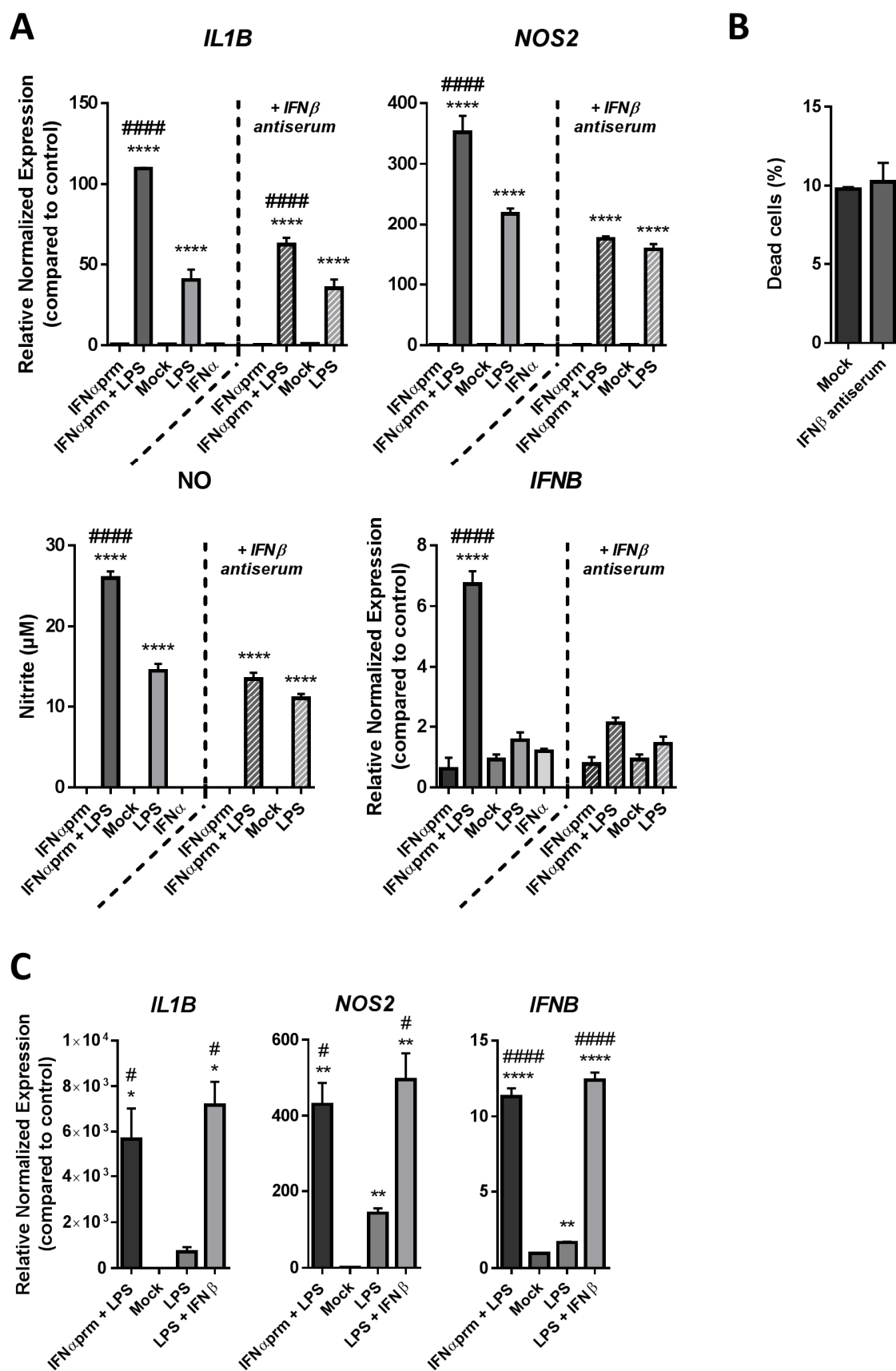
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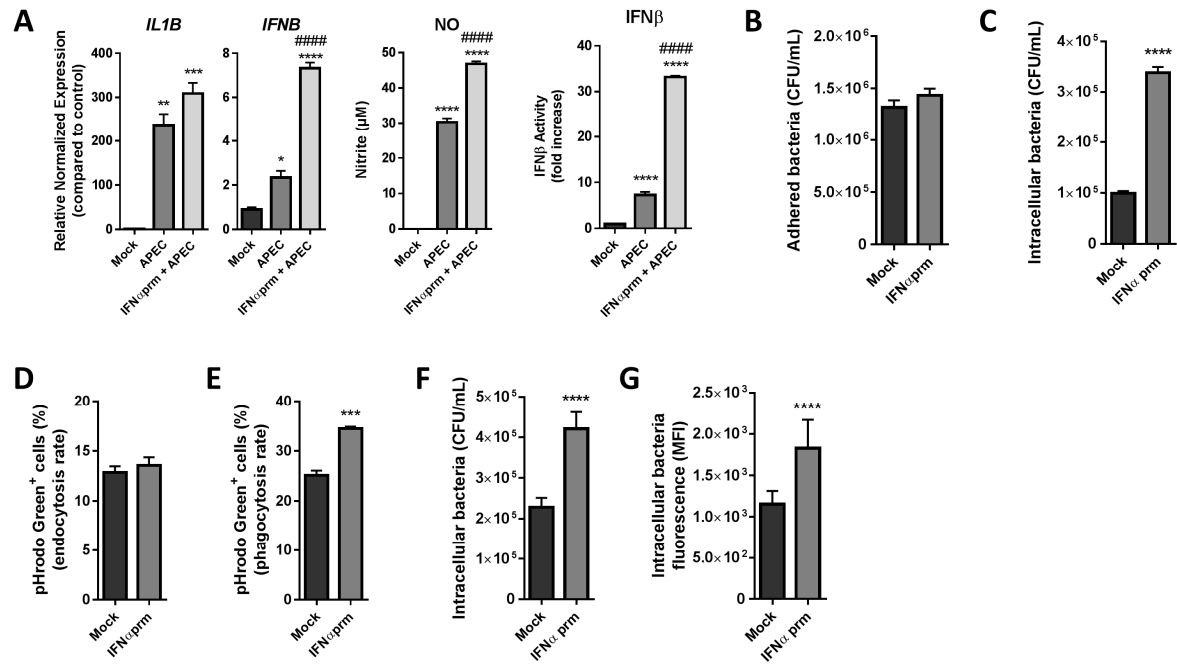
**C**











**Highlights:**

- Type I IFNs differently regulate intracellular events in chicken macrophages
- IFN $\alpha$  priming boosts the macrophage inflammatory response to bacterial challenge
- This boost in the inflammatory response is mediated by IFN $\beta$
- Bacterial uptake is increased if chicken macrophages are primed with IFN $\alpha$